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1. Your reference

REP06103GB

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3. Full name, address and postcode of the or or each applicant *(underline all surnames)*

Medical Biosystems Ltd.
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TQ9 6BX

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

GB

7258635001 DE

4. Title of the invention

SEQUENCING

5. Name of your agent *(if you have one)*

GILL JENNINGS & EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)*

745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor
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c) any named applicant is a corporate body.
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YES

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Continuation sheets of this form	
Description	5
Claim(s)	1
Abstract	
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Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. For the Applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date 6 April 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward
0171 377 1377

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Notes

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SEQUENCING

Field of the Invention

This invention relations to a method for determining the sequence of a polynucleotide.

Background of the Invention

The ability to determine the sequence of a polynucleotide is of great scientific importance. For example, the Human Genome Project is an ambitious international effort to map and sequence the three billion basis of DNA encoded in the human genome. When complete, the resulting sequence database will be a tool of unparalleled power for biomedical research. The major obstacle to the successful completion of this project concerns the technology used in the sequencing process.

The principal method in general use for large-scale DNA sequencing is the chain termination method. This method was first developed by Sanger and Coulson (Sanger *et al*, Proc. Natl. Acad. Sci. USA 1977; 74: 5463-5467), and relies on the use of dideoxy derivatives of the four nucleoside triphosphates which are incorporated into the nascent polynucleotide chain in a polymerase reaction. Upon incorporation, the dideoxy derivatives terminate the polymerase reaction and the products are then separated by gel electrophoresis and analysed to reveal the position at which the particular dideoxy derivative was incorporated into the chain.

Although this method is widely used and produces reliable results, it is recognised that it is slow, labour-intensive and expensive.

An alternative sequencing method is proposed in EP-A-0471732, which uses spectroscopic means to detect the incorporation of a nucleotide into a nascent polynucleotide strand complementary to a target. The method relies on an immobilised complex of template and primer, which is exposed to a flow containing only one of the different nucleotides. Spectroscopic techniques are then used to measure a time-dependent signal arising from the polymerase catalysed growth of the template copy. The spectroscopic techniques described are surface plasmon resonance (SPR) spectroscopy, which measures changes in an analyte within an evanescent wave field, and fluorescence measuring techniques. However, limitations of this method are recognised; the most serious for the SPR technique being that, as the size of the copy strand grows, the absolute size of the signal also grows due to the movement of the strand out of the evanescent wave field, making it harder to detect increments. The fluorescence measuring techniques have the disadvantage of increasing background interference from the fluorophores incorporated on the growing nascent polynucleotide chain. As the chain grows, the background "noise" increases and the time required to detect each nucleotide incorporation needs to be increased. This severely restricts the use of the method for sequencing large polynucleotides.

There is therefore a need for an improved method for determining the sequence of polynucleotides which significantly increases the rate at which a polynucleotide is sequenced and which is preferably carried out by an automated process, reducing the complexity and cost associated with existing methods.

Summary of the Invention

The present invention is based on the realisation that the measurement of electromagnetic radiation can be used to detect a conformational and/or mass change in a helicase and/or primase which occurs when these proteins unwind double-stranded DNA (dsDNA) into single-stranded (ssDNA), using energy from NTP hydrolysis.

According to the present invention, a method for sequencing a polynucleotide comprises the steps of:

(i) reacting a target polynucleotide with a helicase or primase, and if necessary the different nucleotides, under conditions sufficient for enzyme activity; and

(ii) detecting the interaction between the enzyme and a target nucleotide, by measuring radiation.

The radiation may be applied to a sample using a number of techniques, including surface-sensitive detection techniques (in which instance the helicase enzyme will be bound to a solid support), where a change in optical response at a solid optical surface is used to indicate a binding interaction at the surface. In a preferred embodiment of the invention, the technique used is evanescent wave spectroscopy, in particular surface plasmon resonance (SPR) spectroscopy.

Using a helicase in order to determine the sequence of a polynucleotide offers several advantages for the success of this method. Firstly, the problem of secondary structures that exist within polynucleotide molecules is reduced since helicases encounter and overcome these structures within their natural environment. Secondly, helicases offer the ability to directly sequence double-stranded DNA at room temperature. This ability offers advantages in terms of ease of manipulation of target polynucleotides and the possibility of sequencing long polynucleotide templates.

In an embodiment of the invention, the energy available to the helicase, in the form of NTP, is under strict control. That is, the motion of the helicase along the DNA strand to be sequenced is regulated via direct control of the concentration of an energy source molecule in the region of its binding site and hence availability to the helicase molecule. This can allow enzyme activity to be regulated, thereby promoting the measurement of radiation to identify a base or base pair in within proximity to the helicase or helicase complex. One way of controlling the concentration is to use 'caged' or blocked nucleotides that are activated by removal of the block, for example, by photolysis. An example of caged nucleotides is given in WO 99/05315.

Alternatively, the control of DNA unwinding, and hence sequencing progress, may be accomplished by controlling the ability of the helicase enzyme to undergo a conformational change that allows it to either carry out hydrolysis and/or move along a polynucleotide. This may be achieved by engineering (via state-of-the art genetic manipulation techniques) a helicase (or molecule associated with it) so that it contains a chemical/moiety group or groups that enable the helicase to convert or transduce the radiation into a conformational change.

By this means it should be possible to regulate the activity of the enzyme, for example to slow the enzymatic process allowing a distinct signal to be generated for each nucleotide on the target. The selective control of helicase activity is carried out in a way that ensures the detection of each nucleotide. The method may therefore proceed on a real-time basis, allowing base determination and helicase activity to be linked, to achieve a high rate of sequence analysis.

Description of the Invention

The present method for sequencing a polynucleotide involves the analysis of the conformational/kinetic interaction between a helicase enzyme and a target polynucleotide. Measurement of conformational/kinetic interaction is carried out by monitoring the changes in or absorption of electromagnetic or other radiation that occurs if the reaction proceeds.

The term "polynucleotide" as used herein is to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other hybridising nucleic acid-like molecules, e.g. peptide nucleic acid (PNA).

The term "helicase" as used herein is to be interpreted broadly, and relates to ubiquitous proteins that unwind double-stranded polynucleotides into single-stranded polynucleotides, and may or may not utilise energy from NTP hydrolysis to achieve this (Dean, F. B. et al., J. Biol. Chem. (1992) 267:14129-14137; Bramhill, D., & Kornberg., A. Cell (1988) 54:915-918; Schions, M. *et al*, Cell (1988) 52:385-395).

Typically, the method is carried out by applying electromagnetic radiation, by using techniques of surface plasmon resonance or nuclear magnetic resonance. However, other techniques which measure changes in radiation may be considered, for example spectroscopy by total internal reflectance fluorescence (TIRF), attenuated total reflection (ATR), frustrated total reflection (FTR), Brewster angle reflectometry, scattered total internal reflection (STIR) or evanescent wave ellipsometry.

Techniques other than those requiring electromagnetic radiation are also envisaged, in particular photochemical techniques such as chemiluminescence, and gravimetric techniques including resonant systems such as surface acoustic wave (SAW) techniques and quartz crystal microbalance (QCM) techniques.

Surface plasmon resonance (SPR) spectroscopy is a preferred method, and measures the properties of a solution by detecting the differences in refractive index between the bulk phase of the solution and the evanescent wave region. Incident monochromatic light is reflected at a specific angle of a solid optical (sensor chip) surface on the opposite side to the sample under study. The light extends into the sample for a short distance and is affected by an interaction at the surface.

Suitable sensor chips are known in the art. Typically, they comprise an optically transparent material, e.g. glass, and a thin reflective film, e.g. silver or gold. For a review of SPR spectroscopy, see EP-A-0648328.

Nuclear magnetic resonance (NMR) spectroscopy is another preferred method, and measures the magnetic properties of compounds. Nuclei of compounds are energetically orientated by a combination of applied magnetic field and radio-frequency radiation. When the energy exerted on a nucleus equals the energy difference between spin states (the difference between orientation parallel or anti-parallel to the direction of the applied fields), a condition known as resonance is achieved. The absorption and subsequent emission of energy associated with the change from one spin state to the other, is detected by a radio-frequency receiver.

The method of the present invention may be used to determine the sequence of either the DNA strand held within the helicase enzyme during unwinding, or alternatively, maybe used to determine the sequence of the unwinding strand that becomes detached from the helicase.

An important, although not essential, aspect of the present invention is the use of a helicase enzyme/complex immobilised onto a solid support. Immobilisation of the helicase offers several important advantages for the success of this method. Firstly, the problem of random "noise" associated with measuring energy absorption in soluble molecules is reduced considerably. Secondly, the problem of noise from the interaction of any substrate (e.g. NTP sources) not directly involved with the helicase is reduced, as the helicase can be maintained within a specifically defined area relative to the field of measurement. This is particularly relevant if the technique used to measure that changes in radiation requires the measurement of fluorescence, as in TIRF, where background fluorescence increases as the nascent chain grows. Also, if SPR spectroscopy is used, the helicase reactions are maintained within the evanescent wave field and so accurate measurements can be made irrespective of the size of the polynucleotide. Finally, as neither the target polynucleotide nor the oligonucleotide primer is irreversibly attached to the solid surface, it is relatively simple to regenerate the surface, to allow further sequencing reactions to take place using the same immobilised helicase or helicase complex.

Immoblisation may be carried out using standard procedures known is the art. In particular, immobilisation using standard amine coupling procedures may be used, with attachment of ligand-associated amines to, say, a dextran or N-hydroxysuccinimide ester-activated surface. In a preferred embodiment of the invention, the helicase is immoblised onto a SPR sensor chip surface where changes in the refractive index may be measured . Examples of procedures used to immoblise biomolecules to optical sensors are disclosed in EP-A-0589867, and Löfas et al., Biosens. Bioelectron. (1995) 10: 813-822.

The helicase used in the invention may be of any known type. For example, the helicase may be any DNA-dependant DNA helicase, e.g. E.coli DnaB Helicase (Xiong,. Y. et al. J. Mol. Biol. (1996) 259: 7-14.). If the target polynucleotide is a RNA molecule, then the helicase may be a RNA-dependent helicase or a helicase that is able to act on both forms of polynucleotide.

In a preferred embodiment of the invention, the helicase is bacteriophage T7 gp4 helicase (Egelman et al., Proc. Natl. Acad. Sci. USA, (1995) 92:3869-3873). In a further preferred embodiment of the invention, the helicase is either E.coli RuvB helicase (Stasiak et al., Proc. Natl. Acad. Sci. USA, (1994) 91:7618-7622), E.coli DnaB Helicase (Xiong,. Y. et al. J. Mol. Biol. (1996) 259: 7-14.), or simian virus 40 large T helicase (Dean, F. B. et al., J. Biol. Chem. (1992) 267:14129-14137).

In yet another embodiment of the invention, the DNA molecule could be attached to a bead (e.g. one end of the DNA could be biotinylated and attached to a streptavidin-coated polystyrene sphere; Chu, S. Et al, Optical Society of America, Washington, DC, (1990), 8:202). The bead is then held within an optical trap (Ashkin, A., S. Chu. Opt. Lett. (1986) 11:288) within a flow cell. As the helicase (under external control) makes its way along the polynucelotide being sequenced, the polynucleotide can be moved in space via the optical trap (or also known as optical tweezers) to maintain the helicase within the field of detection. It is also envisaged that this system could work in the reverse set-up with a bound helicase being held by the optical trap.

CLAIMS

1. A method for sequencing a polynucleotide, comprising the steps of:

(i) reacting a target polynucleotide with a helicase/primase enzyme, under conditions suitable for enzyme activity; and

(ii) detecting the interaction between the enzyme and a nucleotide on the target, by measuring radiation.

2. A method according to claim 1, wherein the radiation is electromagnetic.

3. A method according to claim 1 or claim 2, wherein step (ii) comprises using surface plasmon resonance.

4. A method according to claim 1 or claim 2, wherein step (ii) comprises using nuclear magnetic resonance.

5. A method according to any preceding claim, wherein the enzyme is immobilised on a solid support.

6. A sensor chip comprising a helicase/primase enzyme immobilised thereon.

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Agent : Gill Jennings & Every